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Therapeutic Potential of Selenium Treated Stem Cells for the Reduction of Liver Fibrosis

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Abstract

Mesenchymal Stem Cells (MSCs) therapy is an alternative way to treat liver fibrosis. The aim of the current study is to enhance the therapeutic potential of MSCs by pretreated with selenium for the reduction of CCl_4 induced liver injury. Male Balb/C mice were treated with CCl4 (1.0 µL/g) intraperitoneally, twice a week for 4 weeks. Mouse MSCs were cultured and then pretreated with 15 ng/ml selenium for 24 hrs. The untreated and selenium pretreated MSCs were transplanted into CCl_4 injured mice. After two weeks of MSCs transplantation, mice were observed for liver regeneration. The morphological result showed that selenium treated MSCs have significant therapeutic effect in reduction of CCl_4 induced injured as compared to untreated MSCs. Biochemical and histopathological result also revealed significant reduction in serum ALT and bilirubin level, collagen content in selenium treated MSCs group as compared to untreated MSCs. Reverse transcriptase PCR result at mRNA level also confirm the antifibrotic effect of selenium treated MSCs on liver fibrosis as evidenced by decreasing the expression level of apoptotic marker and enhancing hepatocyte marker. Thus it is concluded that selenium treated MSCs have a strong therapeutic effect on the reduction of liver fibrosis in CCl_4 mice model.

Keywords: Selenium; Stem cells; Liver fibrosis

Introduction

Liver is a vital organ performing critical functions like urea synthesis, glycogen storage, hormone balance, and detoxification. The liver has an incredible regenerative ability but following chronic liver damage, it begins to fail and eventually develops fibrosis [1]. Liver fibrosis is the wound-healing response of the liver which lead to chronic injury [2]. Chronic carbon tetrachloride (CCl_4) intoxication is a well-known chemical for producing oxidative stress and chemical hepatic injury. Its biotransformation produces hepatotoxic metabolites, the highly reactive trichloromethyl free radicals, which are further converted to the peroxytrichloromethyl radical [3]. Following repeated injury, the liver undergoes tissue remodeling and forms fibrosis, which is characterized by excessive accumulation of extracellular matrix, with the formation of scar tissue encapsulating the area of injury. This results in many clinical manifestations, including ascites, varicella hemorrhage and encephalopathy. The prognosis for patients with the disease is poor, although liver transplantation remains a good alternative treatment. However, there are limited available donor livers for the hundreds of millions of patients worldwide [4,5]. Therefore, it is very necessary to develop an alternative way for the treatments of this disease.

Recently, Mesenchymal Stem Cells (MSCs) has been investigated with the prospect of treatment of acute and chronic liver diseases. Some studies provide clinical and experimental evidence, that MSC transplantation can restore the liver function in acute and chronic damages [5,6]. Mesenchymal Stem Cells (MSCs) are multipotent adult stem cells present in bone marrow, adipose tissue and cord blood and have emerged recently as an attractive candidate for liver repair [7,8]. In the bone marrow, there are main populations of stem cells including hematopoietic stem cells, MSCs and multipotent adult progenitor cells [9]. A number of studies have proven that under appropriate environmental conditions, cells derived from the bone marrow can differentiate into hepatocytes both in vivo [10,11] and *in vitro* [12]. Administration of MSC can decrease liver injury, lungs and heart by reducing inflammation, collagen deposition and rearrangement. Some other reports have shown that transplantation of BMSCs could improve liver fibrosis, but their effects were insignificant [13]. Transplantation of MSCs treated with HGF and found reversal of liver injury in rats [14].

The aim of the present study was to enhance MSCs potential for hepatic repair after CCl_4 induced liver injury in mouse. The current study demonstrated that selenium pretreated MSCs enhance stem cells proliferation and regenerative capacity for the reduction of liver fibrosis as compared to untreated control cells. It was also recognized that selenium (Se) is a strong antioxidant and protects cells from oxidative injury by enhancing the antioxidant activity of glutathione peroxidase and thioredoxin reductase [15]. Studies have also found that selenium has preventive effects in cardiovascular diseases, viral infections, fertility and aging [16-17]. We employed CCl_4 induced liver injury model and observed the ability of pretreated MSCs in reduction of liver fibrosis in-vivo. The present study was undertaken to examine the possible effects of untreated and selenium pretreated MSCs on CCl_4 induced liver fibrosis in mice.

Materials and Methods

Culturing and pre-treatment of MSCs

Mesenchymal Stem Cells(MSCs) were isolated from femur and tibia of (Balb/C) mice according to the protocol described by Khan et al. [18] and were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% fatal bovine serum (FBS, BIOWEST) and 100 U/ml penicillin and 100µg /ml streptomycin (CAPRICON) in the 25 mm culture flask. The culture was maintained in humidified incubator supplied with 5% CO_2 at 37°C. After 70%-90% confluency,

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these cultured MSCs were treated with selenium to enhance their proliferation potency. For this purposes the cells were trypsinized with trypsin (1X). About 1×10^6 cultured MSCs was suspended in serum-free DMEM supplemented with 10% Fetal Bovine Serum and 100 units/ml penicillin and 100 µg/ml streptomycin in a 25 mm flask. The cells were treated with 15 ng/mL of selenium for 24 hrs. Then this selenium treated MSCs were transplanted to fibrotic mice through their tail vain injection.

Preparation of animal model

Six to eight weeks old male albino mice (Balb/C) weighting between 25-30 gm were purchased from pharmacy department, University of Peshawar. The mice were kept in pathogen free environment at constant temperature (20-25°C) with free access to standard rodent diet. In this study four different mice model were prepared which are classified into four groups (five mice in each group). Group I mice (negative control) only received olive oil intraperitoneally twice a week for six weeks. Group II mice (positive control) received CCl4 diluted 1:1 in olive oil intraperitoneally (1 mL/kg) twice per week for six weeks. Group III mice in addition to receiving CCl_4 intraperitoneally (1 mL/kg), received untreated MSCs (1×10^6 cells in 1 mL PBS) by insulin syringe (through tail vain) in the fourth week after CCl4 injection. Similarly, group IV mice in addition to receiving CCl_4 intraperitoneally (1 mL/kg), received selenium treated MSCs (1×10^6 cells in 1 mL PBS) by insulin syringe (through tail vain) in the fourth week after CCl_4 injection. All the experiments were performed according to the guidelines of the ethical committee of Biochemistry Department , Abdul Wali Khan University Mardan, Khyber Pakhtunkhwa Pakistan.

Stem cells transplantation

For MSCs transplantation, first detached the normal and selenium treated cells from culture flask with trypsin (1x) and centrifuge at 5000 rpm for 10 minutes. After centrifugation, dilute the pellet in appropriate amount (100-200 µl) of PBS. Then the diluted pellet was taken in 1 ml syringe and transplant into fibrotic mice through tail vain at a dose of 1×10^6 cells/100 µl PBS/mice.

Euthanasia and tissue harvesting

All groups of mice were sacrificed at 15 days of post-transplantation. At that time, liver tissues and blood were obtained to determine hepatic fibrosis regeneration. The degree of hepatic fibrosis was determined by morphological and histopathological examination of liver, biochemical analysis of blood samples and PCR base analysis of liver RNA.

Biochemical analysis

After euthanasizg the animals by anesthetic chloroform, blood samples were collected from hearts of each group of experimental mouse. Then the blood was centrifuged at 8000 rpm for 10 min to isolate the serum. Serum ALT and bilirubin level were determined through spectrophotometer using the kit (Vitro scient).

Histopathological analysis

After isolation of liver from mice, a segment of liver was fixed in 10% formalin for 24 hours. Then the fixed tissue was processed in a series of ethanol solutions of increasing concentration for dehydration. Then the paraffin sections were prepared and cut into 5-µm sections by a rotary microtome (ROBUS). After bathing in ultrapure water, the sections were taken with the help of microscopic slide and allow to dry at 37°C overnight. Then the sections were stain with hematoxylin (H) and eosin (E) reagents according to standard procedure. The sections were studied through microscope at 10X for histological changes i.e apoptosis and collagen deposition.

PCR analysis

The total RNA from the liver tissue homogenates was isolated using TRizol RNA isolation kit (INVITROGEN). After isolation of RNA, cDNA was synthesized through reverse transcriptase PCR using 2 μg of RNA and oligo-dT primers at 42°C for 60 minutes (Invitrogen kit). Then 100–500 ng/ml of cDNA was amplified through polymerase chain reaction (PCR) using a standard PCR kit. The sequences of the primers were as follows (Table 1). The PCR protocol consisted of 35 cycles at 94∘C for 4 minutes, 56°C–58°C for 45 sec, and 72°C for 30 sec, followed by a final extension step at 72°C for 10 minutes. PCR products were size-fractionated on agarose gels and detected by ethidium bromide staining.

Gene expression levels of hepatic marker (Cytokeratin8) and apoptotic marker (Bax) in all mice model were analyzed by running the PCR product through 1.5% agarose gel. The gel was observed into the gel documentation system under the UV light. The relative expression of target genes was determined by comparing to a reference gene (GAPDH).

Results

Comparative anatomy of liver morphology

The comparative liver morphology of all groups of mice were studied as shown in (Figure 1). The liver morphology of group II mice was more brownish black in color, shrink architecture and scar seen (Figure 1B), as compared to group III and IV. As compared to group II and III, the liver morphology of group IV mice (Figure 1D) was closed to radish black in color with smooth surface and less scar, like group I (Figure 1A). These morphological results revealed that group IV mice showed more similarities to group I as compared to group II and III. Thus group IV mice have more reduction in CCl_4 liver injury as compared to group III (Figure 1C).

Biochemical analysis of liver function

To determine the treatment effect of selenium pretreated MSCs on fibrotic liver, serum level of ALT and bilirubin was analyzed in all groups of mice, using ALT and bilirubin kits (Vitro scient).

Serum ALT value of group II mice (285.5 U/L) showed that there was a significant increase in serum ALT (liver enzyme) level as compared to group I (57.4 units/L) mice. ALT level of group IV mice (127.25 U/L) were significantly lower as compared to group II and group III mice and more closed to group I (57.4 units/L) as shown in the Figure 2A. Similarly, serum bilirubin level was very high in group II mice (1.62 mg/dl) as compared to Group I, III and IV mice. Bilirubin level of group III mice (1.21 mg/dl) showed a slight decrease as compared to group II mice while in group IV mice bilirubin value was 0.83 mg/dl, which was significantly lower than group II and group III mice and close to group I (0.43 mg/dL) mice (Figure 2B). Thus ALT and bilirubin values

PCR primer	Sequence	Annealing temperature	Size in bp
Bax (F) Bax (R)	TGGAGATGAACTGGACAGCA CAAAGTAGAAGAGGGCAACCAC	58°C	152
Cyt-8 (F) Cyt-8 (R)	CTCACTAGCCCTGGCTTCAG ACAGCTGTCTCCCCGTGA	57°C	232
GAPDH (F) GAPDH (R)	CTCTTGCTCTCAGTATCCTTG GCTCACTGGCATGGCCTTCCG	57°C	372

Table 1: List of primer with their sequence, annealing temp and product size.

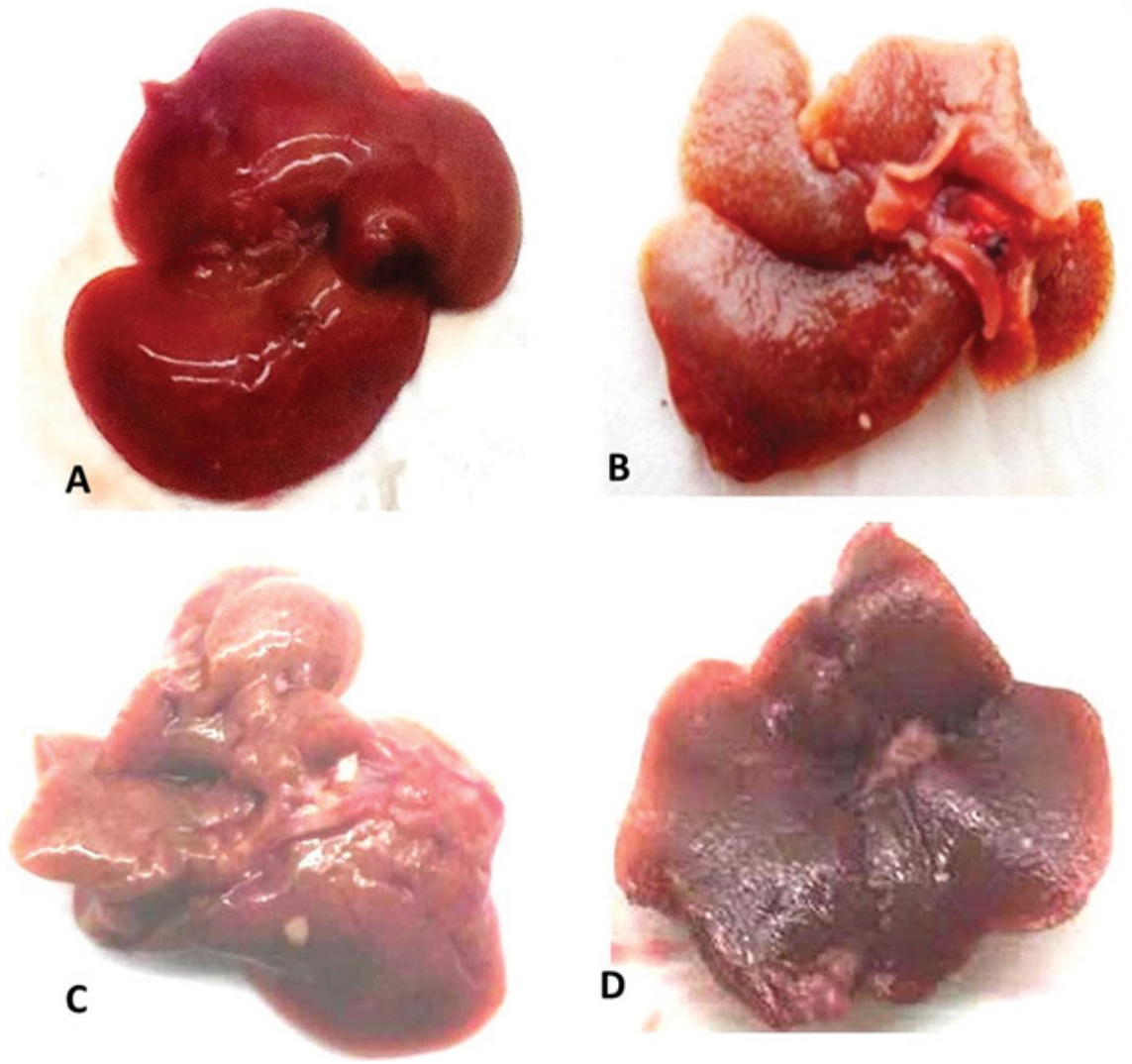

Figure 1: Comparative anatomy of liver morphology of group I (A), Group II (B), Group III (C), Group IV (D) mice.

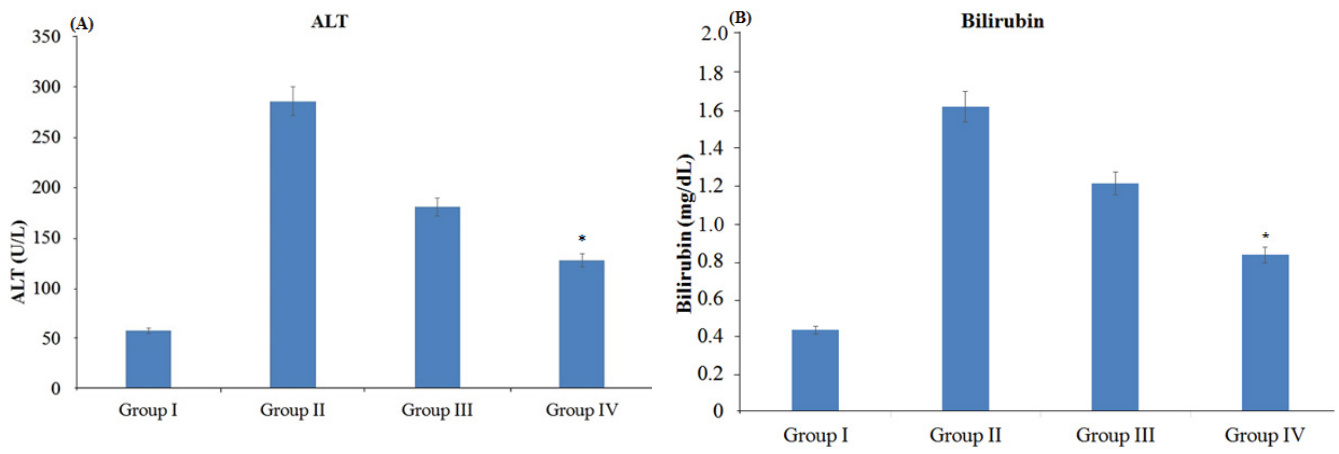

Figure 2: Liver function analysis by studding serum ALT (A) and bilirubin (B) level changed in CCl_4 untreated and pre-treated MSCs transplantation.

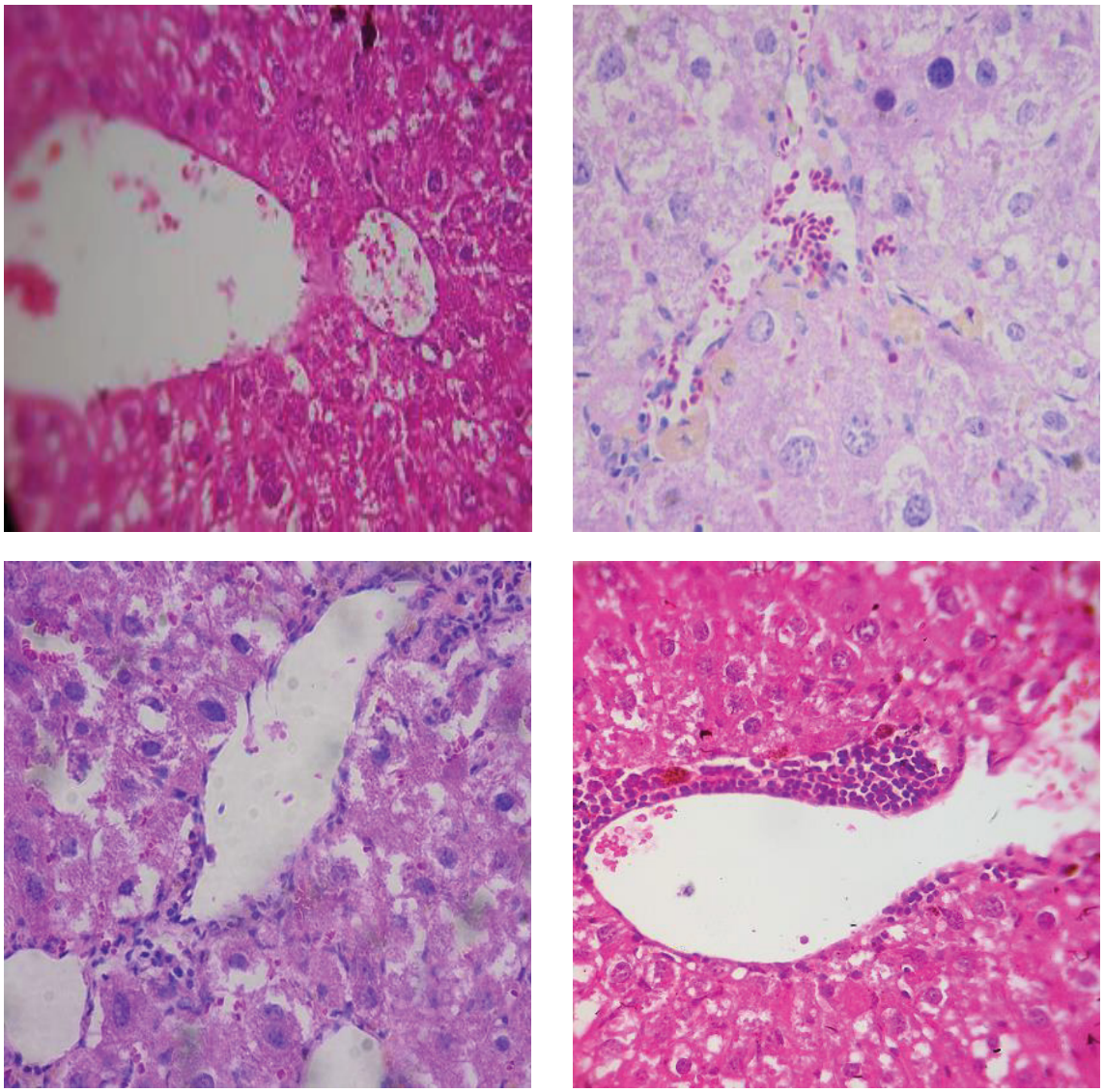

Figure 3: Effect of untreated and selenium treated MSCs on histopathological changes induced by CCl_4 in mice (10X). A: (group I), B: (group II), C: (group III), D: (group IV).

of group IV mice were more closed to group I, which clearly indicated that selenium pretreated MSCs have high recovery on hepatic function as compared to untreated MSCs.

Effect of MSCs transplantation on apoptosis

The liver section (H &E staining) of group II mice have large number of apoptotic hepatocyte, more central vain congestion and high liver collagen (Figure 3B) as compared to group I mice. Mice treated with untreated MSCs (Group III) showed reduce apoptosis, less central vain congestion and also less collagen deposition (Figure 3C), as compared to group II mice. However Figure 4D showed that the liver section of selenium treated MSCs mice (Group IV) have significant antifibrotic effect as evidenced by a significant reduction in liver collagen, less central vain congestion and also less number of apoptotic hepatocytes as compared to group II and III. Thus comparative study in the Figure 3 showed that the liver section of group IV mice was more similar to group I as compared to group II and III.

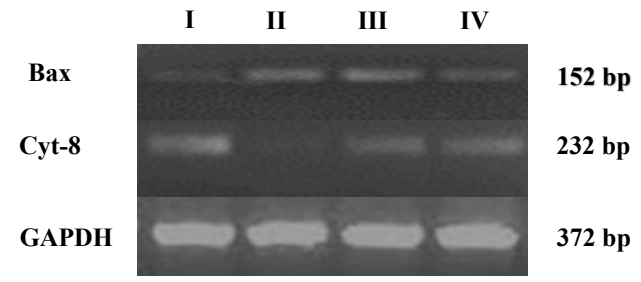

Figure 4: Expression of Bax, Cyt-8 and GAPDH: I; group I mice, II; group II mice, III; group III mice, IV; group IV mice.

Gene expression analysis

The expression level of apoptotic and hepatocyte markers in all experimental groups of mice were analyzed by using reverse

transcriptase PCR at mRNA level. In this study GAPDH is used as an internal control. The expression of Bax marker was down regulated in group IV as compared to group II and III as shown in the Figure 4. One the other hand, the expression level of Cyt-8 marker was upregulated in selenium treated MSCs transplanted mice. Thus Cyt-8 marker showed increase expression in group III as compared to group II but significant increase was observed in group IV mice compared to group II and III (Figure 4).

Discussion

Liver fibrosis is healing response to chronic liver injury in which mostly accumulation of Extracellular Matrix (ECM) proteins occur [5]. In this disease fibrotic scar is produced which is basically composed of type I and III fibrillar collagen, fibronectin and proteoglycans [19]. It has been investigated that MSCs transplantation can restore normal function of liver in acute and chronic liver disease [5,6]. In this study bone marrow derived MSCs culture was pretreated with 15 ng/ml selenium for 24 hrs. These selenium pretreatment' enhance the proliferation capacity of MSCs culture and cause significant therapeutic effect on liver fibrosis in CCl4 injured mice.

Glutathione (antioxidant) plays a very significant role in the detoxification of hydrogen peroxide, other peroxides and free radicals and thus provides the reduction capacity for most reactions [20]. The hepatic content of glutathione in CCl_4 treated rats was observed to be extensively decreased as compared to the controls. MSC-based therapy significantly increased hepatic glutathione content in the MSC-treated group and completely stop their inhibition (Ayatollahi et al.). It has been investigated that selenium treated MSCs therapy have more antioxidant effect on CCl4 treated mice as compared to untreated MSCs therapy. Selenium treated MSCs recovered GSH content to normal level and in this way stop the progression of liver fibrosis.

In 2013 Rungruang et al. reported that the liver morphology of normal mice was reddish black in colors whereas the liver morphology of disease mice brownish-black in colors [21]. Therefore the morphological result of Rungruang et al. [21] strongly supported the current study results. The liver morphology of group II mice (Figure 1B) was brownish black in color whereas the liver morphology of group IV mice (Figure 1D) showed more similarity to group I (Figure 1A) and radish black in color as compared to group II and III mice (Figure 1B and 1C). This morphological result showed that selenium treated MSCs have significant antifibrotic effect on CCl4 as compared to untreated MSCs.

The liver also contains many important enzymes for their biological function such as biological degradation and further detoxification of harmful substances. CCl4 treatment increases the activity of AST and ALT in plasma with lipid accumulation and necrosis in the hepatocyte rate [22]. In this study, the effect of untreated and selenium pretreated MSCs was investigated at serum bilirubin and ALT level in mice liver injured with CCl4. MSCs transplantation (Group III) restored the increase level of liver enzymes in serum as compared to group II but a significant reduction of ALT and bilirubin was observed to normal level as compared to both group II and III (Figures 2A and 2B). This restoration of serum ALT and bilirubin to normal level by selenium treated MSCs transplantation indicate normal function of liver. Recovery of liver function after MSCs transplantation was also examined by histopathologically. MSCs reversed the hepatic necrosis, fatty changes, and inflammation [23]. The histopathological examination of CCl_4 livers exhibited a significant increase in liver collagen surrounding the hepatic lobules with central vain congestion and apoptotic hepatocyte (Figure 3B). In the Figure 3D selenium treated MSCs have significant antiapoptotic effect on liver apoptosis. Group IV mice have low number of apoptotic hepatocyte due to strong antioxidant effect of selenium as compared to group III (Figures 3C and 3D).

PCR base analysis also revealed that the expression of apoptotic markers such as Bax and caspase-3 was high in CCl_4 treated hepatocyte while the expression of hepatocyte markers such as albumin was also inversely proportional to the concentration and duration of CCl_4 treatment [24]. All these markers are the indicator that clearly demonstrates liver fibrosis. These indicators were reversed after transplantation of untreated and selenium treated MSCs to CCl_4 injured mice. The result showed, that the expression level expression of Bax marker was low in group IV mice, whereas the expression level of cyt-8 marker in group IV mice was significantly high as compared to group II and III as shown in the Figure 4. Thus it is cleared that selenium treated MSCs have high antiapoptotic and antifibrotic capacity as compared to untreated MSCs to reduce liver fibrosis in CCl_4 injured mice. Thus from all these results it was finally concluded that selenium pretreated MSCs have high regenerative ability for fibrotic liver. Transplantation of these cells can restore the normal function of fibrotic liver.

Conclusion

The present study suggested that selenium treated bone marrow derived MSCs have strong regenerative capability on the reduction of liver fibrosis on CCl_4 injured mice. In conclusion, due to active proliferation of MSCs with selenium and their strong antioxidant effect, the selenium treated MSCs produced a complete reversion in CCl_4 induced fibrotic liver by decreasing hepatic collagen content and enhancing regenerative capability of hepatocyte. Therefore selenium treated MSCs transplantation enhanced the liver function by reducing fibrosis in CCl_4-induced liver fibrotic mice. Thus from morphological, biochemical, histopathological and RT-PCR result it was concluded that selenium treated MSCs transplantation have more significant therapeutic effect on liver damage, particularly for those due to oxidative stress, as compared to untreated MSCs.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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